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Received June 12, 1962. P.S.E.B.M., 1962, v111.

A Regulated Incubator Controlling CO_2 Concentration, Humidity and Temperature for Use in Animal Cell Culture.* (27707)

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Clonal plating of mammalian cells requires regulation of temperature, humidity and CO_2 concentration within narrow limits. Although small experiments can be carried out in sealed desiccators or plastic bags containing appropriate gas mixtures, operation on a larger scale requires an incubator which can be opened and closed repeatedly without fluctuations in temperature or gaseous composition.

An incubator which is continuously flushed with a humidified carbon dioxide-air mixture has been described elsewhere(1), and has been successfully employed for several years. However, such incubators depend on a constant rate of delivery of clean, compressed air, which is frequently not available. Moreover, even with the highest rates of gas flow which are practicable, recovery of the steady state conditions after opening of the incubator door is slow enough to cause serious difficulties in a heavily used incubator.

For these reasons, a new incubator was designed, in which the CO_2 concentration is continuously monitored, and pure CO_2 is injected automatically as needed to maintain the desired level. Water vapor is continuously supplied by a humidifier located inside the incubator. The arrangement is illustrated in Fig. 1 and 2.

* This work was supported by grants from The National Foundation and Damon Runyon Memorial Fund for Cancer Research, Inc.

Principle of operation. Carbon dioxide concentration is monitored by measuring the pH of a standardized sodium bicarbonate solution through which gas from the incubator is continuously passed. The pH meter operates a switching circuit which causes the injection of additional CO_2 whenever the concentration falls below a predetermined level.

The following equipment is needed for construction of the carbon dioxide control apparatus shown in Fig. 2. (The apparatus as shown is designed for use with a double-door, water jacketed incubator with 2 thermometer ports in its top, and with sufficient space between its left and right side shelves for the blower duct system. Application to other incubators will require some alteration of the design. The numbers refer to the location of items in Fig. 2.)

1. "Standard" tank of liquid CO_2 (delivers gaseous CO_2), National Cylinder Gas Medical Grade, or equivalent.

2. Two stage automatic regulator. Matheson No. 8, with No. 320 connection.

3. Low pressure shutoff valve (supplied as part of item 2).

4. Solenoid valve. Skinner V52DA2100 or equivalent. Normally closed. ¼″ NPT connections.

5. Combination needle valve and flow gauge. Hoke style 993, with meter 2142. 0.2 - 1.5 l.p.m., calibrated for CO_2.

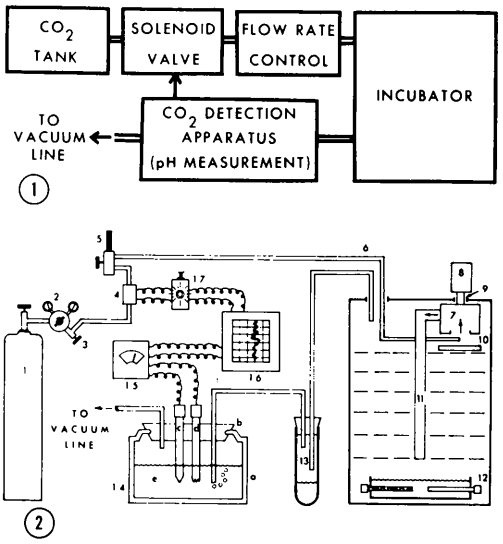

FIG. 1. Flow diagram for Controlled CO_2 Incubator.

FIG. 2. Details of Controlled CO_2 Incubator. The sizes of condensation trap (13) and CO_2 detecting cell (14) have been greatly exaggerated to show detail.

6. Carbon dioxide injection line. ¼″ OD "Nylaflow" nylon tubing (Polymer Corp. of Pennsylvania), threaded through a port of the incubator, and terminating directly under the blower intake. (This type of tubing is used throughout the apparatus, with the exception of the condensation trap. "Nyloseal" compression fittings (Nalge Corp.) are used as needed for connection to ¼″ NPT threaded equipment.)

7. Blower. Eastern Air Devices J57C, 110 CFM, modified by placing extension shaft and housing between blower body and motor, and by connecting a duct system to the output. Intake and output are indicated by arrows.

8. Blower motor, placed outside incubator to protect it from humidity, and to prevent heating effects.

9. Extension shaft and housing connecting motor to blower through another port of incubator.

10. Flat metal baffle placed about one inch below blower intake to break up air currents.

11. Duct system, carrying blower output toward bottom of incubator, terminating about 1½″ above water pan.

12. Stainless steel water pan containing 125 watt knife heater (Central Scientific Co.) wired in series with thermostat (Fenwal Thermoswitch, Hexhead type). The thermostat is adjusted so that the heater is turned on when temperature of water falls below the normal operating temperature of the incubator. Heater and thermostat are installed at right angles, such that they come within 2 inches of meeting directly under the blower duct outlet.

13. Condensation trap: Test tube fitted with 2-hole stopper and 5 mm glass tubing, connected to nylon tubing with short lengths of rubber tubing.

14. CO_2 detecting cell. a) Wide mouth 4 oz. jar. (Van Waters and Rogers, Cat. No. 16195, or equivalent) Must fit No. 12 stopper. b) No. 12 silicone rubber stopper. (West Co.) Stopper is cut to one-half normal thickness with a sharp knife, and a narrow groove is cut around the outer surface to insure a more secure fit in the jar. Holes for electrodes and tubing are cut by pushing cork borers straight through stopper without twisting. c) Glass pH electrode. (Leeds and Northrup No. STD-1199-30) d) Reference electrode. (Leeds and Northrup No. STD-1199-31) Care must be taken to position electrodes so that they will not be damaged by contact with the bottom of the jar. Reference electrode must also be positioned such that its vent is not covered by the stopper. e) Indicator solution. $NaHCO_3$ 1.2 g per liter, Phenol Red, 0.0012 g per liter in distilled water. Jar is filled about one-half full.

15. pH meter. (Leeds and Northrup 7664 or 7401 with 20 ohm output resistor).

16. Recorder with 3 switching circuits (one for CO_2 control, and the other 2 for high and low alarms). (Leeds and Northrup Cat. No. 3-930-051-133-6-001 Speedomax H model S indicating and recording controller. 0-5 millivolt range).

17. Pilot light indicating when solenoid valve for CO_2 addition is activated and push button switch for manual addition of CO_2.

In normal operation, gas from the incubator is continually aspirated through the pH cell. The rate of bubbling is not critical, although very slow rates will lead to sluggish

response, and unduly rapid rates may cause excessive condensation of water vapor into the cell. Excessive humidity must be removed from the gas sample before it enters the pH cell to prevent condensation and dilution of the indicator solution, which would lead to falsely acidic pH readings, and result in maintenance of the CO_2 concentration at too low a level. One to three or more test tube condensation traps (item 13, Fig. 2) connected in series may be required for effective control of this situation. The initial level of the indicator solution is marked on the outside of the cell, so that slight changes in its volume can be easily detected. The solution should be replaced whenever its volume changes appreciably, or whenever it develops cloudiness or precipitate. The phenol red in the indicator solution provides a quick visual check on the function of the apparatus.

The CO_2 sampling line must not be constricted, since this will cause an increased vacuum and therefore a decreased partial pressure of CO_2 in the detecting cell, which will result in excessive CO_2 addition.

Humidity control is accomplished by forced circulation of the incubator's atmosphere over the water in the pan at the bottom of the incubator. Whenever the humidity in the incubator falls, the water is cooled by the resulting increased evaporation. The water pan thermostat then turns on the water heater, until the operating temperature is restored. This arrangement leads to rapid recovery of both humidity and temperature after the incubator door has been opened. A humidistat or humidity recorder can be added, but experience with the present design has shown these not to be necessary.

A sensitive thermostat placed in the incubator's water jacket, and connected in series with the regular air thermostat of the incubator is used for temperature control. The air thermostat is set approximately one degree high, so that it opens the circuit only in case of failure of the water thermostat. (For details, see reference 1.) A warning light is wired in parallel with the air thermostat. As long as the air thermostat remains closed, the lamp is bypassed, but it becomes activated if the air thermostat opens while the water jacket thermostat is still calling for heat.

The CO_2 detector which has been described will respond to a change from zero CO_2 concentration to the normal level of 5% in well under 5 minutes. Thus, the rate of CO_2 addition may be adjusted to provide rapid recovery without danger of overshooting the end point because of detector lag. In practice, a CO_2 addition rate of 1.5 liters per minute into an incubator of approximately 200 liters capacity has been found to give recovery to within 0.2—0.3 pH unit of the desired level, in 5 to 7 minutes, and complete recovery in 10 to 15 minutes, with no detectable overshooting. Complete humidity recovery is also obtained within 10 to 15 minutes.

The second and third switching circuits on the recorder are used for an alarm system to indicate failure of the CO_2 control apparatus. They are set 0.1 pH unit above and below the control point, and are connected through a 20 minute time delay switch to an alarm bell. Such a system will not respond to the normal control cycling of pH, which is less than $\pm$ 0.1 unit, nor to opening of the incubator door, since complete recovery occurs in less than 20 minutes. However, a major malfunction of the CO_2 control system will cause the alarm to sound before serious damage has been done to the cells in the incubator.

The limiting factor in the precision of control of CO_2 concentration in the incubator is the long-term stability of the pH meter and electrodes. On restandardization of the meter with standard buffer solution, the variation is generally observed to be less than $\pm$ 0.1 pH unit, and has never been more than $\pm$ 0.2 pH unit, during a period of several months.

In calculating the relationship between the meter pH and CO_2 content of the incubator, several factors must be considered. The basic equations can be written as follows:

$$pH = pK + \log_{10}(HCO_3^-) - \log_{10}(H_2CO_3)$$

$$\text{and } (H_2CO_3) = (S)(F)(P_A - P_W),$$

where $pK = 6.09$, $\log_{10}(HCO_3^-) = -1.85$,

S = solubility of CO_2 in water, expressed as moles per liter per mm Hg partial pressure of CO_2

F = mole fraction of CO_2 in a dry air-CO_2 mixture
P_A = Atmospheric pressure
P_W = Vapor pressure of water.

Since the incubator is maintained at 37.5° C, while the detector cell is normally set on top of the incubator and operated at approximately 30°C, it is necessary to make separate detector and incubator pH calculations for a given CO_2-bicarbonate mixture. At 37.5°C, $S = 3.1 \times 10^{-5}$M/l/mm Hg and $P_w = 48$ mm Hg, while at 30°, the values are 3.8×10^{-5} and 32. Since atmospheric pressure enters into the equations, it is necessary to make altitude corrections. In Denver, where the apparatus was tested, the atmospheric pressure is only 625 mm Hg, compared to a standard sea level value of 760 mm Hg.

When the above equations are solved for Denver and 37.5°C, the relationship between pH and mole fraction CO_2 is:

$$pH = 5.99 - \log_{10}F.$$

The correction for sea level is –0.09 pH unit, and the correction for 30°C is –0.10 pH unit. Thus, for example, 5% CO_2 (F = .05) corresponds to pH 7.29 in Denver at 37.5°C, to 7.19 in Denver at 30°, to 7.20 at sea level and 37.5°, and 7.10 at sea level and 30°.

The bicarbonate concentration selected for use in the indicator solution is the same as that in the cell culture media routinely used in the incubator(1). Thus, it is possible to calculate the pH of the equilibrated media inside the incubator simply by adding a temperature correction of 0.1 pH unit to the meter value.

In practice, the apparatus is set to maintain a meter pH of 7.20, which corresponds to an incubator pH of 7.30, or approximately 5% CO_2 in air. At this concentration, 0.1 pH unit corresponds roughly to one per cent carbon dioxide. Thus, the apparatus is capable of maintaining the CO_2 level at 5% with a long term accuracy of about ± 1% CO_2 concentration.

Certain precautions must be rigidly observed to insure long term stable operation of the unit. These include: 1) The KCl solution in the reference electrode must be checked regularly and replenished as needed; 2) the condensation traps must be emptied regularly; 3) the CO_2 tank must be changed as needed so that the supply is never completely exhausted; 4) the water pan must be kept filled.

It is recommended that these details be checked at least twice a week. If the condensation traps are found to require attention too frequently, a larger bottle can be put in place of the first test tube. However, the size should be kept small, since increasing the trap volume slows the response time of the CO_2 detector.

In an alternate setup, the same basic CO_2 regulating system may be added to a continuous-flow gassing system and be used only to speed recovery when the incubator door has been opened. When this is done, the blower is not strictly necessary. However, in the absence of a blower, it is desirable to locate the CO_2 sampling and CO_2 injection lines such that there is some pickup of newly injected CO_2 by the detector. This is necessary to avoid overshooting due to slow mixing of the newly injected CO_2 with the contents of the incubator. Such an arrangement does not result in as precise control as the apparatus in Fig. 2. However, it has an advantage of being composed of 2 independent systems, either of which is capable of maintaining the incubator during an emergency period, and it does overcome the problem of slow recovery, which is the worst defect of the continuous gassing system.

The CO_2 control apparatus can also be attached to non-humidified chambers. However, in such cases, it is necessary to saturate the gas sample with water vapor prior to its entry into the detector in order to avoid evaporation of the indicator solution.

A simple, reliable CO_2 control system makes the study of the effect of CO_2 concentration on a variety of biological systems much more convenient. With the apparatus described above, CO_2 concentration may be altered and accurately maintained at a new value simply by changing a pointer setting.

It has been demonstrated(2) that indefinite growth of euploid mammalian cells without change in chromosomal constitution can be achieved, but only with careful control of environmental conditions, *i.e.*, the composition

of the nutrient medium, and the temperature, humidity and p_{CO_2}. Several media have been developed for this purpose. With the combination of the latest and best of these media (3) and the incubator described here, growth of euploid cells has been more rapid and reliable than under any other conditions of our experience.

Summary. 1. A device for continuously monitoring and regulating the carbon dioxide content of cell culture incubators is presented. 2. CO_2 concentration is determined by measuring the pH of a bicarbonate solution which is continuously equilibrated with gas samples drawn from the incubator. 3. Installation and operation of the device in a practical cell culture incubator is described.

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Received June 15, 1962. P.S.E.B.M., 1962, v111.

Inhibitory Effect of Ammonium Sulfate on Columbia SK Virus Propagation in Mouse Ascites Tumor Cells *in vitro.* (27708)

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While screening antiviral substances by using an adapted strain of Columbia SK virus which can propagate efficiently in mouse ascites tumor cells(1,2,3,4), it was found that ammonium sulfate showed a definite inhibition against the cytopathic effect, hemagglutinin production, and replication of infectious virus. Several workers(5,6,7,8) have reported the inhibitory effect of ammonium ions on the influenza virus group in tissue cultures but have found no effect on other viruses. We now show that Columbia SK virus also is sensitive to this simple ion.

Materials and methods. A hemagglutination (HA)-positive strain and HA-negative strain of Columbia SK (Col SK) virus which are adapted to Ehrlich ascites cells were used in such cells. In some experiments, LCM virus (strain NY#621) which was isolated from a leukemic L_2C guinea pig by Dr. C. W. Jungeblut(9) was used, also in Ehrlich cells. After washing, 2 to 4 $\times$ 10^6 Ehrlich ascites cells harvested freshly from mice were suspended in 1 ml of culture medium per tube. The medium was composed 85% of Medium 199 and 15% of heat-inactivated (60°C, 30 min) Ehrlich ascites fluid from which fibrin had been removed by standing at 4°C for a week. Virus was added as 0.05 ml of a definite dilution of virus with Medium 199; ammonium compounds were added as 0.05 ml of aqueous solutions. The same volumes of Medium 199 and of distilled water were added to controls. The stationary cultures were incubated at 36°C. After various incubation times, cultures were shaken by hand to remove the cells from the glass wall, and then centrifuged. The supernatant was used for titration of infectivity and HA. The cells were resuspended in saline and stained with 1 drop of 0.4% of erythrosin which colors the damaged cells red but does not stain living cells. With this staining method, the cytopathic effect (CPE) of the virus and the toxicity of the chemicals could be detected quantitatively with a counting error within 10%. More than 75% loss of live cells, as compared with the controls, was recorded as 4+; 74% to 50% as 3+; 49% to 25% as 2+; and under 24% as 1+. For HA, a 0.2% suspension of sheep red cells in potassium barbital buffer was used(10). The infectivity was determined in culture tubes as previously described(3).

Results. 1. *Effect of ammonium salts on multiplication of virus with standard inoculum size.* An inoculum of 0.05 ml of 10^{-4} dilution, about 1000 tissue culture doses